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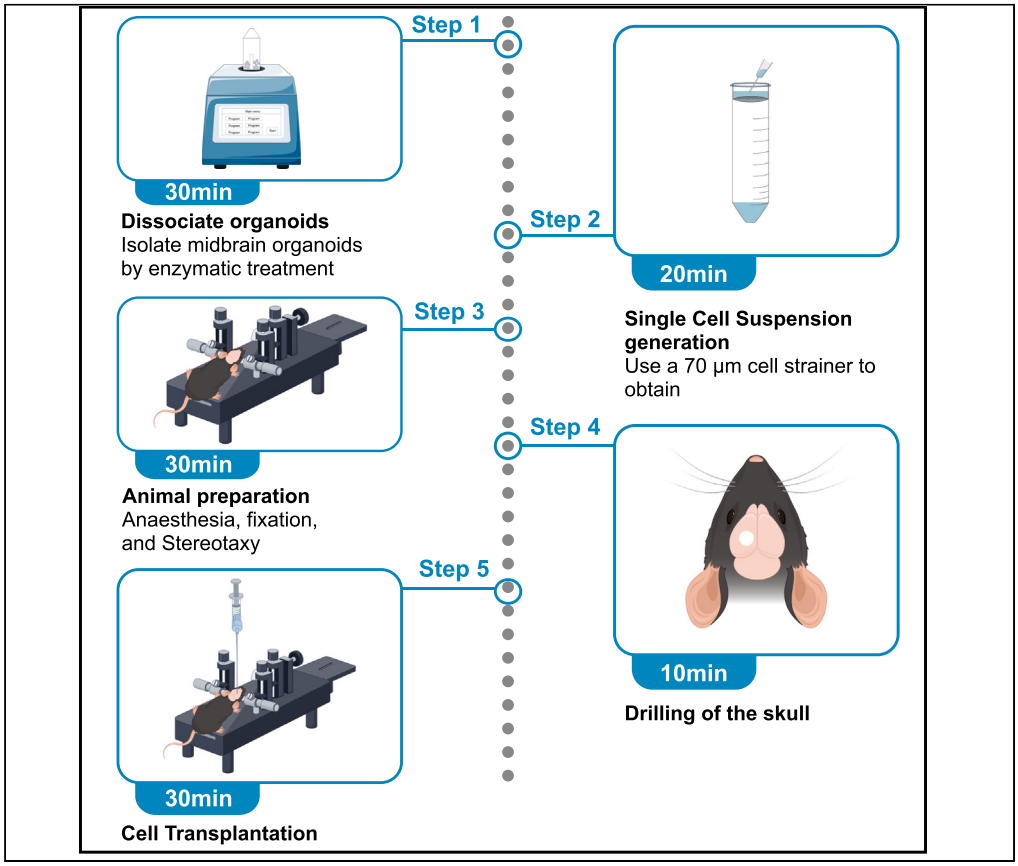
Protocol for transplantation of cells derived from human midbrain organoids into a Parkinson's disease mouse model to restore motor function



Midbrain organoids provide an innovative cellular source for transplantation therapies of neurodegenerative diseases. Here, we present a protocol for midbrain organoid-derived cell transplantation into a Parkinson's disease mouse model. We describe steps for midbrain organoid generation, single-cell suspension preparation, and cell transplantation. This approach is valuable for studying the efficacy of midbrain organoids as a potential cellular source for restoring motor function.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Preparation of human iPSC-derived midbrain organoids

Steps for isolating midbrain organoids by enzymatic treatment

Step-by-step guide for transplanting cells from midbrain organoids into a PD mouse model

Fu et al., STAR Protocols 5, 103251
September 20, 2024
https://doi.org/10.1016/j.xpro.2024.103251

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Protocol for transplantation of cells derived from human midbrain organoids into a Parkinson's disease mouse model to restore motor function

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https://doi.org/10.1016/j.xpro.2024.103251

SUMMARY

Midbrain organoids provide an innovative cellular source for transplantation therapies of neurodegenerative diseases. Here, we present a protocol for midbrain organoid-derived cell transplantation into a Parkinson's disease mouse model. We describe steps for midbrain organoid generation, single-cell suspension preparation, and cell transplantation. This approach is valuable for studying the efficacy of midbrain organoids as a potential cellular source for restoring motor function.

For complete details on the use and execution of this protocol, please refer to Fu et al.[1]

BEFORE YOU BEGIN

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the degeneration of dopaminergic neurons in the substantia nigra, leading to motor dysfunction. Cell transplantation, especially using midbrain organoid-derived cells, has emerged as a potential therapeutic approach for PD.[2] This protocol aims to provide the specific steps for midbrain organoid-derived cell transplantation into a PD mouse model to restore motor function.

This protocol outlines the generation of midbrain organoids, employing a methodology established and detailed in an earlier publication.[3] The technique has proven effective for organoids cultivated from induced pluripotent stem cells (iPSCs) and those from embryonic stem cell lines. For an expansive review of the cell lines tested with this protocol, our preceding research should be consulted.[4,5] While the principal application of this protocol is geared towards the engraftment of cells derived from midbrain organoids into the striatum of rodents, modifications may allow for its application in transplantations to alternative regions.

Note that the organoid techniques described in this protocol require operators with extensive stem cell experience. In addition, the surgical procedures described in this paper involve advanced techniques and need to be performed by operators with extensive experience in rodent aseptic surgery using specialized neurosurgical instruments.

Institutional permissions

All the experimental procedures were approved by the Institutional Animal Care & Use Committee of Tsinghua University and the Animal Welfare and Ethics Committee of Tsinghua University

(Approval ID: 22-YJ1). The animals were paired and kept in controlled environments, with a consistent 12-h photoperiod starting at 7:00 a.m. They had unrestricted access to food and water, and utmost measures have been taken to alleviate discomfort.

Human iPSC-derived midbrain organoid preparation

Timing: 30 days

1. Prepare Matrigel-coated plates.
   a. Transfer 12 mL of DMEM/F-12 media into a 15 mL tube and maintain it on ice.
   b. Take 200 μL of the chilled DMEM/F-12 and combine it with a 100 μL frozen Matrigel.
   c. Utilize a pipette to repeatedly mix the solution to facilitate the thaw and dissolution of the Matrigel.
   d. Subsequently, return the mixture to the initial tube containing the chilled DMEM/F-12 and thoroughly blend the contents.
   e. Promptly apply this mixture to coat the surfaces of tissue culture-treated plates, ensuring even distribution by gently swirling each plate. Allow the coated plates to equilibrate for a minimum of 2 h prior to utilization, ensuring they remain adequately hydrated.
2. Maintain hiPSCs on Matrigel-coated plates in a PSC media or E8 media. hiPSC thawing/culture and routine passaging (cell density/division ratio) can be referred to published papers.[6]

⚠ CRITICAL: For quality control, hiPSC can proliferate stably and rapidly for a long time, have clonal morphology and gene expression highly similar to human ES cells, maintain a normal karyotype for a long time, and have the potential of triple-germ layer differentiation *in vitro* and *in vivo*. The alkaline phosphatase activity of hiPSCs is good, and the cells are passaged 1:2 to 1:4, two to three times a week. Cell purity is high, and >90% of the cells expressed SOX2 protein.

3. Prepare embryoid bodies (EBs).
   a. Dissociate hiPSC colonies with collagenase IV (1 mg/mL) at 37°C for 30 min, plate onto low attachment plates and let stand in the incubator for 24 h.
   b. Supplement the PSC with EB media. EB media contained the ROCK inhibitor, which needed to be removed after 24 h.
   c. From this step, transfer the low attachment plate on the orbital shaker placed in the incubator at a speed set to 70 rpm. Culture for 4 days to form EBs (full media changes, 2 mL media for one hole of the 6-well plate, every other day).

Note: The advantage of seeding EBs with collagenase IV is the higher survival rate of EBs. Although it can lead to differences in EB size, this difference has little effect on downstream differentiation and transplantation. In addition, EBs can be prepared by filtering or using Aggrewell 800 (Stem cell, Cat#27965) to obtain uniform size EBs. When observed under light microscopy, EBs are dense spherical, regular in shape, with smooth edges and no bulges.

4. Transfer the EBs into SHH media for 2 days.
5. At day 7, transfer the organoids into an induced media for 7 days (full media changes, 2 mL media for one hole of the 6-well plate, every other day).
6. At day 15, transfer the organoids into a differentiation media for 15 days to obtain the required organoids (full media changes, 2 mL media for one hole of the 6-well plate, every other day).

⚠ CRITICAL: Immunofluorescence staining can be used to ensure the EBs have floor plate cells and dopaminergic neurons. Representative images of EBs at each stage and staining protocol can refer to our previous study.[1] Approximate 80% FP cells and 20% DA neurons

is the acceptable composition. During differentiation, EBs require continuous culture with no pause points where they can be frozen.

PD mouse model preparation

Timing: 4 weeks

7. Use adult male mice at 7 weeks of age for the 6-OHDA-lesioned model.
8. Inject 3.6 μg of 6-OHDA per mouse into the medial forebrain bundle (MFB) region in the left hemisphere, using an ice-cold saline solution.[7]

⚠ CRITICAL: The concentration of the 6-OHDA is 15 mg/mL, always use fresh preparation solution, on the ice, 3 h of use!

9. Calculate the coordinates in reference to the bregma as follows: anterior-posterior (A/P) at −1.2 mm, medio-lateral (M/L) at 1.3 mm, and dorso-ventral (D/V) at 4.75 mm.

⚠ CRITICAL: The injection site is the left hemisphere of the brain, not the right hemisphere. The surgical procedure and injection rate for 6-OHDA were consistent with the details provided in the subsequent sections.

10. House all mice in groups of four per cage at a constant temperature environment under a 12 h:12 h light/dark cycle (7:00 to 19:00) for 4 weeks. Ensure water and food are freely available.
11. Behavioral experiments and immunofluorescence staining are needed to confirm the lesions in mice and to select mice acceptable for transplantation of organoid derived cells, refer to our previous study.[1]

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-mouse Alexa Fluor 488	Jackson ImmunoResearch	Cat#715-545-151; RRID: AB_2341099; 1:500
Goat anti-rabbit Alexa Fluor 647	Invitrogen	Cat#A-31573; 1:500
Mouse anti-STEM121	Cellartis	Cat#Y40410; RRID: AB_2801314; 1:300
Rabbit anti-NeuN	Abcam	Cat#ab177487; RRID: AB_2532109; 1:300
DAPI	Sigma	Cat#D9542; 1:10000
Experimental models: Cell lines		
Human: iPSC line derived from healthy fibroblasts (male)	The Jun Yao Laboratory	Cat#HC-M-C7
Experimental models: Organisms/strains		
Mouse: C57BL/6J (wild type, male, 7 weeks of age, male)	The Jackson Laboratory	Strain #:000664, RRID: IMSR_JAX:000664
Chemicals, peptides, and recombinant proteins		
6-OHDA	Topscience	Cat#28094-15-7
PFA	Sangon	Cat#A500684
Tris	Amresco	Cat#77-86-1
Triton X-100	Biodee	Cat#0694
Adhesive	3M Vetbond	Cat#1469SB
Accutase	Invitrogen	Cat#00-4555-56
B27 supplement	Thermo Fisher Scientific	Cat#17504-044
BDNF	PeproTech	Cat#450-02
cAMP	Sigma	Cat#A6885

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
CHIR 99021	Cellagen Tech	Cat#C2447-2s
DMEM/F12	Thermo Fisher Scientific	Cat#11330-032
EDTA	Thermo Fisher Scientific	Cat#15575-038
FGF-8	PeproTech	Cat#100-25A
GDNF	PeproTech	Cat#100-25
GlutaMAX	Thermo Fisher Scientific	Cat#35050-061
L-ascorbic acid	Sigma	Cat#A92902
LDN-193189	STEMCELL	Cat#72146
Matrigel	Corning	Cat#354234
N2 supplement	Thermo Fisher Scientific	Cat#17502-048
Neurobasal media	Thermo Fisher Scientific	Cat#21103-049
Pen/Strep	Thermo Fisher Scientific	Cat#15140-122
PSCeasy media	Cellapybio	Cat#CA1014500
E8 media	Thermo Fisher Scientific	A1517001
Purmorphamine	STEMCELL	Cat#72202
Y27632	Enzo Life Sciences	Cat#ALX-270-333
SB-431542	Cellagen Tech	Cat#C7243-5
SHH	PeproTech	Cat#100-45
TGF-β	PeproTech	Cat#100-21
2-Mercaptoethanol	Thermo Fisher Scientific	Cat#21985-023
Collagenase IV	Thermo Fisher Scientific	Cat#17104-019
Neural tissue dissociation kit	Miltenyi Biotec	Cat#130-092-628
Mice adult brain tissue mild enzymolysis kit	RWD	Cat#DHABE-5003
Other		
Stereotaxic instrument	RWD	Cat#68045
Neuros syringes	Hamilton	Cat#65460-03
gentleMACS Octo dissociator	Miltenyi Biotec	Cat#130-095-937
Single-cell suspension preparation apparatus	RWD	Cat# DSC-800

MATERIALS AND EQUIPMENT

EB media		
Reagent	Final concentration	Amount
Y27632 (10 mM)	10 μM	50 μL
SB-431542 (10 mM)	10 μM	50 μL
SHH (250 μg/mL)	100 ng/mL	20 μL
LDN-193189 (10 mM)	100 nM	0.5 μL
Purmorphamine (5 mM)	2 μM	20 μL
FGF-8 (250 μg/mL)	100 ng/mL	20 μL
PSCeasy media	N/A	50 mL
Total	**N/A**	**50 mL**

Small molecules/factors are stored in a −80°C freezer for up to 12 months, and media is stored at 4°C for up to 1 month.

SHH media		
Reagent	Final concentration	Amount
LDN-193189 (10 mM)	100 nM	5 μL
CHIR99021 (10 mM)	3 μM	150 μL
SHH (250 μg/mL)	100 ng/mL	200 μL
Purmorphamine (5 mM)	2 μM	200 μL
FGF-8 (250 μg/mL)	100 ng/mL	200 μL
GlutaMax	1 ×	5 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
N2	1 ×	5 mL
DMEM/F12	N/A	490 mL
Total	**N/A**	**500 mL**

Small molecules/factors are stored in a −80°C freezer for up to 12 months, and media is stored at 4°C for up to 1 month.

Induced media		
Reagent	Final concentration	Amount
CHIR99021 (10 mM)	3 μM	150 μL
LDN-193189 (10 mM)	100 nM	5 μL
GlutaMax	1 ×	5 mL
N2	1 ×	5 mL
DMEM/F12	N/A	490 mL
Total	**N/A**	**500 mL**

Small molecules/factors are stored in a −80°C freezer for up to 12 months, and media is stored at 4°C for up to 1 month.

Differentiation media		
Reagent	Final concentration	Amount
2-Mercaptoethanol	0.1 mM	900 μL
BDNF (20 μg/ mL)	20 ng/mL	500 μL
GDNF (200 μg/ mL)	20 ng/mL	50 μL
L-ascorbic Acid (0.2 M)	0.2 mM	500 μL
cAMP (50 mM)	0.5 mM	5 mL
TGF-β (10 μg/mL)	1 ng/mL	50 μL
GlutaMax	1 ×	5 mL
B27	1 ×	5 mL
Neurobasal	N/A	500 mL
Total	**N/A**	**500 mL**

Small molecules/factors are stored in a −80°C freezer for up to 12 months, and media is stored at 4°C for up to 1 month.

STEP-BY-STEP METHOD DETAILS

Dissociate midbrain organoids

Timing: 30 min

The aim of this step is to isolate midbrain organoids by enzymatic treatment. Refer to the instructions of Neural Tissue Dissociation kit for further details. For organoid selection, at about day 30 of midbrain organoids development, DA progenitor cells and DA neurons accounted for the highest proportion of cells, while oligodendrocytes and astrocytes accounted for a very low ratio. The required organoids have a diameter of about 100 μm, regular shape, smooth edges, and no bumps.

1. Determine the weight of organoids in 1 mL of HBSS (Figures 1A and 1B).

   Note: This step requires at least about 30 organoids. Prior to formal experiments, it is recommended to perform pre-experiments to determine cell yield.

2. Prepare enzyme mix 1 by mix up 50 μL enzyme P and 1900 μL buffer X. Transfer 1950 μL enzyme mix 1 into a single cell tube (Figure 1C).

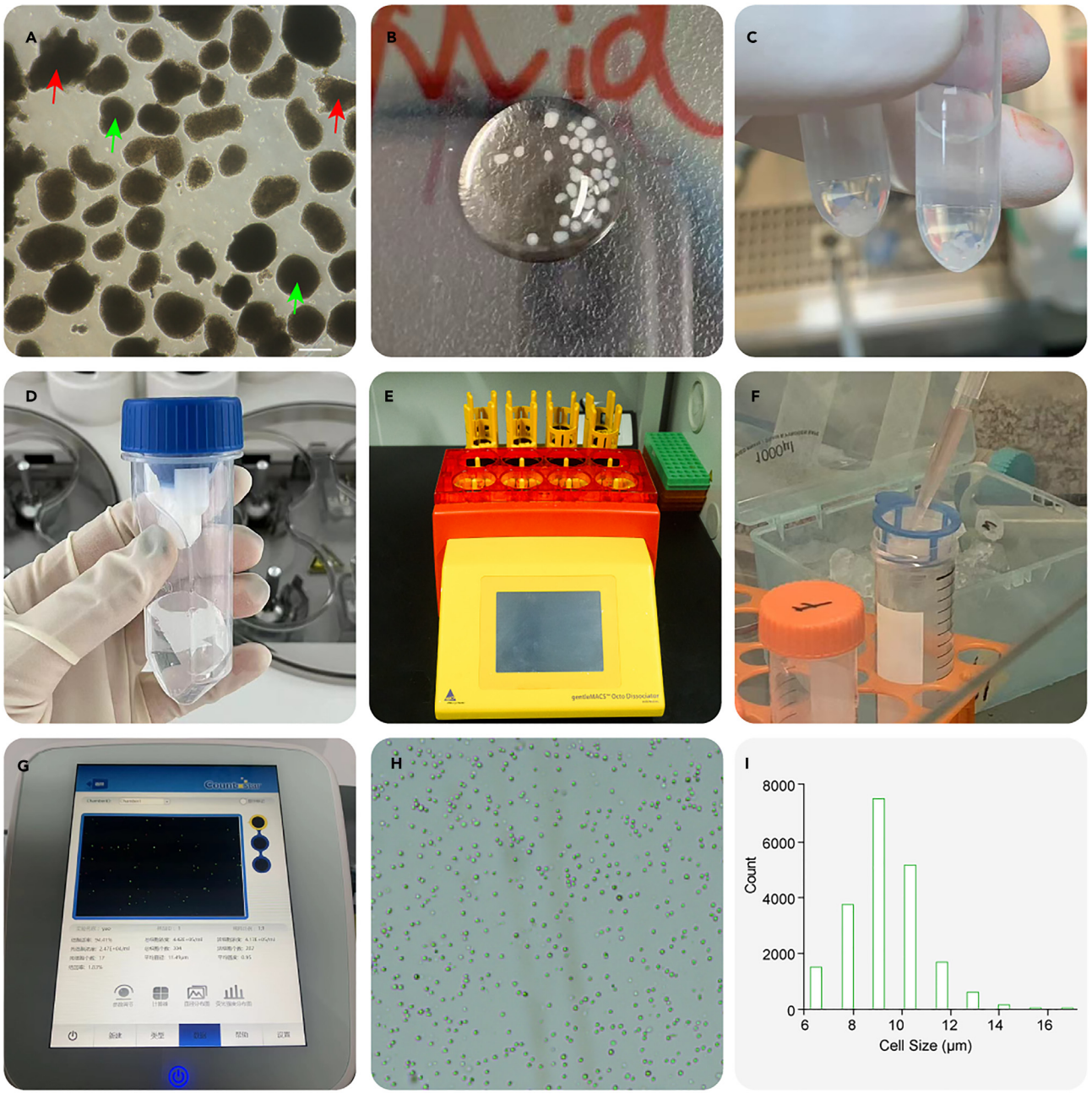

Figure 1. Dissociation of midbrain organoids and generation of single-cell suspension

(A–C) Representative images of midbrain organoids. Green arrows indicate well-differentiated organoids, while red arrows indicate poor-differentiated organoids. Scale bar, 100 μm.

(D) Transfer the enzyme mix into a single cell tube.

(E) Place the single cell tube upside down on the sleeve of the gentleMACS Octo Dissociator.

(F) Transfer the cell suspension to a 70 μm cell filter placed on a 50 mL tube.

(G) Count the cells and check for viability and clumping rate.

(H) Representative image of single-cell suspension.

(I) Statistical plot of single cell size. Data are represented as values.

3. Transfer up to 400 mg midbrain organoids into the single cell tube.
4. Prepare enzyme mix 2 by mix up 10 μL enzyme A and 20 μL buffer Y. Transfer 30 μL enzyme mix 2 into the single cell tube.

5. Close the single cell tube tightly and place it upside down on the sleeve of the gentleMACS Octo Dissociator.
6. Run the Program 37C-NTDK-1 (Figure 1D).

Single-cell suspension generation

Timing: 20 min

The aim of this step is to obtain the single-cell suspension.

7. Remove the single cell tube from the dissociator after the program has finished.
8. Centrifuge the sample briefly to collect it at the bottom of the tube.
9. After resuspending the sample with 1 mL HBSS, transfer the cell suspension to a 70 μm cell filter placed on a 50 mL tube (Figure 1E).
10. Add 10 mL of HBSS through the cell filter to wash the cells.
11. Centrifuge the cell suspension at 300 × *g* for 10 min, then completely aspirate the supernatant.
12. Resuspend the cells in the 1X PBS + 0.04% BSA solution to the required volume for further applications.

Note: The cells should be placed on ice until the animal is prepared. Once midbrain organoids have been prepared into single-cell suspensions, cell debris can be removed using a cell debris removal kit to improve cell purity, but this step is not required.

13. Count the cells and check for viability and clumping rate (Figure 1F).

Note: Transplantation is recommended immediately after preparation of the single-cell suspension to further reduce cell death. Percentage of dead cells before transplantation can accept less than 20%. The acceptable percentage of aggregates in single-cell suspensions was below 10%. If the cell survival rate is too low, it is suggested that early experiments many times, shorten the operation time, in order to improve the survival rate. If the aggregate rate is too high, it is recommended to repeat filtering step.

14. Adjust the concentration of the cell suspension to $2–5 \times 10^4$ cells per μL.

Animal preparation

Timing: 30 min

This step involves induction of anesthesia, fixation of the animal to the stereotaxic apparatus and disinfection of the surgical site to ensure that the animal is maintained in a stable condition to facilitate subsequent surgical procedures. For xenotransplantation in immunocompetent animals, it is important to perform immunosuppression. By daily intraperitoneal injection of Cyclosporine A (10 mg/kg) to inhibit T-cell activation and interleukin-2, to improve the success rate of transplantation.

15. Weigh the mouse to determine its body weight.
16. Anesthetize the mouse using 1% sodium pentobarbital at a dosage of 40 mg/kg body weight.

Note: Prepare the sodium pentobarbital solution with physiological saline and sterilize by filtration through a 0.22 μm filter.

17. Position the anesthetized mouse smoothly on the work surface.
18. Secure the mouse's teeth onto the tooth bar and tighten the nose clip to hold the head steady.

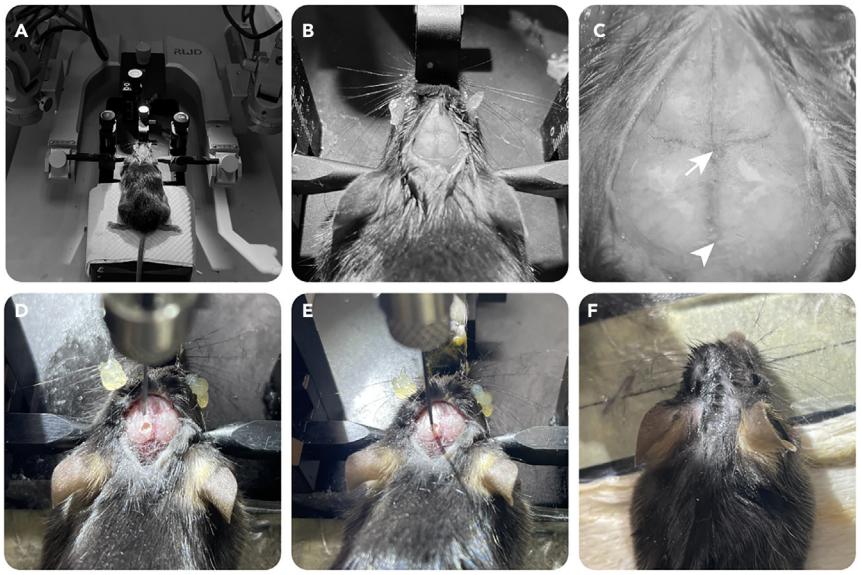

Figure 2. Animal preparation and cell transplantation

(A–C) Anesthetize and fix the animal to the stereotaxic apparatus. The arrow indicates the position of point bregma, and the arrowhead indicates the position of point lambda.

(D) Drilling of the skull.

(E) Inject the single-cell suspension into the formulated brain area.

(F) Suture the skin.

19. Insert ear bars into the external auditory canals of the mouse, ensuring that the scales on both sides are aligned to fix the head position.
20. Adjust the body height of the mouse moderately so that the animal's body and head remain horizontal, and then secure it in place.
21. Apply erythromycin ophthalmic ointment to the animal's eyes.
22. Swab the animal's head with povidone-iodine solution.
23. Use ophthalmic scissors to remove the hair from the head.
24. Incise the skin on the head and peel away the periosteum.
25. Wipe with a cotton swab to fully expose the intersection of the coronal and sagittal sutures, known as the bregma.
26. Adjust the XY axes to position the tip of the microinjection needle at the bregma.
27. Set the coordinates at the bregma as zero.
28. Move the XY axes to bring the microinjection needle tip to the lambda, then read and record the stereotaxic coordinates for the XYZ axes.

Note: Lambda is located in the posterior fontanelle is the meaning of the word intersection seam and sagittal suture.

29. Compare the difference in height between the bregma and lambda.

Note: A difference within 0.03 mm is considered level in the anteroposterior direction of the skull.

30. Adjust 2.3 mm laterally from the bregma on both sides and compare the height difference (Figures 2A–2C).

Note: A difference within 0.03 mm is considered level in the left-right direction of the skull.

Stereotaxy and drilling of the skull

Timing: 10 min

This step aims to determine the injection site and to punch a hole in the skull.

31. Calculate the coordinates for the injection site within the target nucleus, such as the Caudate Putamen (CPu), with AP = 0.5 mm; ML = 2.0 mm; DV = 3.0 mm, based on the mouse brain atlas.[8]
32. Adjust the XY axes to position the tip of the microinjector needle at the designated site.
33. Mark the site with a marker pen.
34. Drill a hole at the marked site using a cranial drill.

Note: When performing a craniotomy, pay attention to the depth of the drill hole in the skull. Too deep is easy to cause excessive blood loss and reduce the survival rate of mice.

35. Use a cotton swab dipped with iodophor to clear away the cranial debris (Figure 2D).

Cell transplantation

Timing: 30 min

This step aims to inject the single-cell suspension into the formulated brain area.

36. Mount the microsyringe onto the stereotaxic apparatus.

Note: Giving preference to 5 μL Hamilton Neuros syringes (Cat#65460–03). During transplantation, first injecting needles into slightly deeper than 3.0 mm (DV), and then move back and create a larger cavity for transplanted cells.

37. Operate the microinjection pump to slowly aspirate 2 μL of the single-cell suspension.
38. Gently insert the needle of the microsyringe, loaded with the cell suspension, into the targeted brain area to the specified depth.
39. Allow the apparatus to rest for 2 min.
40. Inject at a rate of 1 μL/10 min, with a total injection volume of 1 μL (Figure 2E).
41. After completing the injection, let the system rest for 5 min.

Postoperative care

Timing: 10 min

42. Gradually elevate the needle of the microsyringe.
43. Approximate the skin edges with forceps, apply biological tissue adhesive, and hold stationary for 10 s to bond the skin (Figure 2F).

Note: Alternatively, suture the skin using surgical needles and absorbable sutures.

44. Swab the mouse's head with povidone-iodine solution to disinfect the area.
45. Release the ear bars and nose clip, then carefully remove the mouse.
46. Place the mouse on a heating pad to allow for natural recovery from anesthesia.
47. The analgesic was meloxicam, and 4 mg/kg/day was injected subcutaneously when the mice woke up after surgery.
48. Depending on the recovery status of the animals, the drug was given every day for about 3 days.

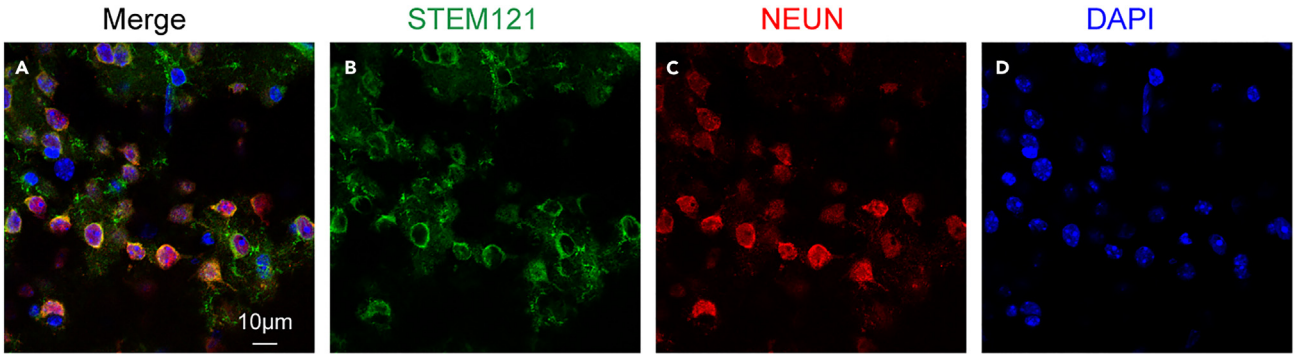

Figure 3. Transplanted cell visualization after animal sacrifice

(A–D) Immunofluorescence staining of the human-specific cell marker STEM121 (B), neuron marker NeuN (C), and cell nucleus marker DAPI (D) in grafted region. Scale bar, 10 μm.

Transplanted cell visualization

Timing: 4 days

This step aims to detect the presence of transplanted cells.

49. Perform cardiac perfusion on the mouse following the standard protocol.
50. Dissect the mouse's brain in its entirety.
51. Submerge the mouse brain in a 30% sucrose solution for adequate dehydration.
52. Section the dehydrated brain tissue by cryostat into slices of 40 μm thickness.
53. Execute immunofluorescent staining according to the standard protocol, utilizing the STEM121 antibody to label the transplanted cells (Figures 3A–3C).

Note: For immunofluorescent staining, the primary antibodies used were mouse anti-STEM121 antibody (1:300), and rabbit anti-NeuN monoclonal antibody (1:300). The secondary antibodies used were donkey anti-mouse Alexa Fluor 488 (1:500), and donkey anti-rabbit Alexa Fluor 647 (1:500). The permeabilization buffer used is 0.5% Triton-X 100 in PBS. The primary and secondary antibodies are diluted in 5% BSA in PBS. The dilution and incubation time for DAPI are 1:10000 and 15 min. More details about staining can refer to Fu et al., 2024.[1]

EXPECTED OUTCOMES

At the day30, the midbrain organoids enriched DA progenitor cells and DA neurons, included 79% FP cells, 20% DA neurons, and 1% vascular leptomeningeal cells, astrocytes, and oligodendrocyte progenitors. The concentration of the cell suspension is $2–5\times10^4$ cells per μL. Upon completing this experimental protocol, the transplanted cells, derived from human midbrain organoids, are anticipated to integrate structurally and functionally with the host brain in an effective manner. The survival status of the transplanted cells can be visually ascertained by applying of immunofluorescent staining techniques. The expected survival rate of the transplanted cells is about 80% or more. The functional integrity of the transplanted neurons can be corroborated by employing virus-mediated labeling combined with dopamine probe assessments. In the absence of complications, postoperative maintenance of the animal subjects may extend beyond six months.

LIMITATIONS

The utilization of the Neuronal Tissue Dissociation Kit from Miltenyi Biotec for the digestion of midbrain organoids necessitates the employment of specialized instrumentation, specifically the gentleMACS Octo Dissociator with Heaters, which limits the widespread application of this protocol.

Employing of alternative organoid digestion protocols is a potential strategy to circumvent this limitation. The mice adult brain tissue mild enzymolysis kit (RWD, Cat#DHABE-5003) and the Single-cell suspension preparation apparatus (RWD, Cat#DSC-800) from RWD company are good alternatives.

TROUBLESHOOTING

Problem 1

Insufficient procurement of midbrain organoids has been observed for the purpose of digestion.

Potential solution

The issue may be attributed to an inadequate supply of iPSCs utilized for differentiation; it is advisable to augment the initial cellular quantity. The initial seeding density of iPSC is recommended to be greater than 1 million cells per well and 1:3 passage. It is available when grown to about 80% of the culture surface bottom area. The purity of D30 organoids should be at least 50%, and 80% is best. In our experience, 1 well of a 6 well plate of hiPSCs would give us 20–30 EBs, and 30 EBs would typically give us 15–20 organoids on D30, which would be enough for 1–2 transplantations.

Problem 2

The acquired single-cell suspension manifests a viable cell count less than 2×10^4 cells per μL.

Potential solution

This issue may be precipitated by a multiplicity of factors. It is plausible that the selected midbrain organoids have already undergone partial necrosis, containing an excessive number of dead cells; attention should be directed toward selecting midbrain organoids in a satisfactory state. Alternatively, the problem could stem from a procedural error, resulting in insufficient digestion time; it is advisable to meticulously examine the programming settings of the apparatus. Good and poor organoids usually appear in the same batch of differentiation, which is inevitable. In our experience, these buds/irregularly shaped organoids usually appear after 7 days. When there are more than 50% of the poor organoids in a batch, we recommend not to continue differentiation to ensure the ultimate success rate.

Problem 3

Subsequent to the craniotomy, there was a substantial increase in hemorrhagic output.

Potential solution

The issue in question may be attributable to an excessive penetration depth of the cranial drill during the craniotomy procedure, resulting in damage to the cerebral vasculature. It is imperative to meticulously regulate the drilling depth of the cranial drill.

Problem 4

The mice died after transplantation.

Potential solution

The complication in question could be attributed to either an overdose of anesthesia or excessive hemorrhage during the surgical procedure. Alternatively, it may also arise from accidental injury to other cerebral regions during the operation. The dosage of 1% pentobarbital sodium is 40 mg/kg, and the mice must be weighed in advance to avoid excessive anesthesia.

Problem 5

No viable transplanted cells were observed.

Potential solution

It is conceivable that a heightened aggregation rate of the cells may have led to the occlusion of the injection needle, thereby precluding the successful administration of the cells.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jun Yao (jyao@mail.tsinghua.edu.cn).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Chong-Lei Fu (fuchonglei@163.com).

Materials availability

This protocol does not generate new materials.

Data and code availability

All data reported in this protocol will be shared by the lead contact upon request, and this protocol does not report original code.

ACKNOWLEDGMENTS

We thank all members of the laboratory for helpful discussion and technical assistance. This work was supported by the Beijing Municipal Natural Science Foundation (grant no. Z210011), the National Natural Science Foundation of China (grant nos. 31771482 and 31830038), and the Shandong Postdoctoral Science Foundation (SDBX202302023 and SDCX-ZG-202400157). The graphical abstract of this protocol includes cartoon components sourced from www.figdraw.com (ID: WPRRP33e65).

AUTHOR CONTRIBUTIONS

C.-L.F. and J.Y. developed and modified this protocol. C.-L.F. wrote this manuscript with the help of J.Y. X.J., B.-C.D., D.L., and X.-Y.S. performed and analyzed the experimental component of this protocol. All the authors edited and approved this manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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